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A Human Liver Cell Line Exhibits Efficient Translation of HCV RNAs Produced by a Recombinant Adenovirus Expressing T7 RNA Polymerase

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An *in vitro* system that supports the efficient growth of hepatitis C virus (HCV) and reflects its complete *in vitro* replication cycle has not yet been established. The establishment of a minigene RNA of HCV in mammalian cells could facilitate the study of virus–cell interactions and the molecular pathogenesis of this virus. We constructed a replication-deficient recombinant adenovirus expressing bacteriophage T7 RNA polymerase under the control of CAG promoter (AdexCAT7). A high level of T7 RNA polymerase was detectable for at least 11 days after inoculation. Cells infected with AdexCAT7 were then transfected with plasmids carrying the authentic T7 promoter, the 5′ untranslated region (UTR) of encephalomyocarditis virus, a luciferase gene, and a T7 terminator (pT7EMCVLuc) or carrying the modified T7 promoter, the 5′UTR of HCV, a luciferase gene, the coding region of C-terminal of NS5B and the 3′UTR of HCV, a ribozyme of hepatitis D virus and a T7 terminator (pT7HCVLuc). Most of the cell lines examined supported a higher expression of luciferase by transfection with pT7EMCVLuc than with pT7HCVLuc. However, one cell line, FLC4, derived from a human hepatocellular carcinoma, exhibited very high reporter gene expression with pT7HCVLuc. In this cell line, transfection with RNA synthesized *in vitro* from pT7HCVLuc induced a higher level of reporter gene expression than RNA from pT7EMCVLuc. The T7-adenovirus system for the synthesis of HCV minigenes *in vivo* provides useful information on the molecular mechanisms of HCV translation in human liver cells.

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of posttransfusion and sporadic non-A, non-B hepatitis. HCV has a high propensity to cause chronic hepatitis, which may progress to liver cirrhosis and to hepatocellular carcinoma (Saito *et al.,* 1990). HCV is distantly related to pestiviruses and flaviviruses, and its genome consists of approximately 9400 nucleotides (nt) of positive-strand RNA encoding a single polyprotein of 3010–3033 amino acids (Kato *et al.,* 1990; Choo *et al.,* 1991; Takamizawa *et al.,* 1991).

The genomic structure of HCV, including both the 5′ and 3′ termini, has been well characterized. The mechanisms of processing of the HCV polyprotein have been investigated by analyses of cDNA expression *in vivo* and *in vitro* under the control of foreign promoters using different vectors (Trono *et al.,* 1988; Hijikata *et al.,* 1991; Matsuura *et al.,* 1992; Grakoui *et al.,* 1993). However, authentic HCV protein may not be identical to these recombinant cDNA-derived HCV proteins.

Although some lymphocyte cell lines have been shown to support partial replication of HCV (Shimizu *et al.,* 1992; Kato *et al.,* 1995), there are currently no efficient *in vitro* systems yet to grow HCV. Recently it was reported that a full-length HCV RNA transcribed from a cDNA clone of HCV was infectious for a chimpanzee by direct injection into his liver (Kolyhalov *et al.,* 1997; Yanagi *et al.,* 1997). However, propagation of the infectious RNA in cell culture has not been reported so far.

Recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase (VacT7) has been used for studies on processing of HCV polyproteins (Grakoui *et al.,* 1993; Selby *et al.,* 1993). This system was further applied for recovering infectious viruses from their full-size cDNA clones of negative-strand RNA viruses like, rabies virus, vesicular stomatitis virus, respiratory syncytial virus, Sendai virus, and bunyavirus (Trono *et al.,* 1988; Conzelmann and Schnell, 1994; Collins *et al.,* 1995; Garcin *et al.,* 1995; Lawson *et al.,* 1995; Bridgen and Elliott, 1996; Kato *et al.,* 1996). However, extensive cytopathic effects (CPE) caused by replication of a vaccinia virus vector sometimes interfere with a long-term analysis of the expressed products (Wyatt *et al.,* 1995) and impede infectious virus recovery from their cDNAs.

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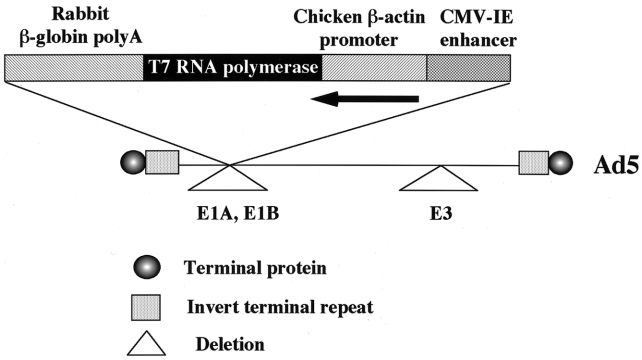

FIG. 1. Structure of AdexCAT7. The bacteriophage T7 RNA polymerase gene under the control of CAG promoter was inserted into the E1A and E1B deletion region of the adenovirus genome. The arrow shows the orientation of the transcription.

The object of this study was to establish a system to facilitate the analysis of the mechanism of HCV replication *in vivo.* To this goal, a replication-deficient recombinant adenovirus expressing T7 RNA polymerase under the control of the CAG promoter (AdexCAT7) was made. It circumvents the CPE caused by replication of the vector virus. To establish a system to examine the translation efficiency of HCV minigene RNA *in vivo,* a plasmid harboring a luciferase gene flanked by 5′ and 3′ untranslated regions (UTR) of HCV under the T7 promoter was also made. Following transfection of AdexCAT7-infected cells with the plasmid, the HCV minigene RNA carrying the 5′ and 3′UTR of HCV at both ends of the luciferase gene was efficiently synthesized *in vivo.* We examined various mammalian cells for luciferase expression from the minigene and found that one human hepatoma cell line supported efficient expression of HCV minigene RNA. The HCV minigene system using this cell line is useful to study the virus-cell interaction of HCV infection and other viruses for which there are no efficient *in vitro* replication systems.

RESULTS

Construction of AdexCAT7

AdexCAT7 was constructed by cotransfecting 293 cells with the pAdexCAT7 and *Eco*T22I-digested DNA-terminal protein complex of adenovirus type 5 genome through homologous recombination (Fig. 1). Purified AdexCAT7 and a control virus AxCAwt were obtained at titers of 3×10^9 and 4×10^{10} plaque-forming units/ml, respectively.

Expression of T7 RNA polymerase in various mammalian cells infected with AdexCAT7

Various mammalian cells were infected with AdexCAT7. Cell lysates were examined for the polymerase activity by the incorporation of [^{3}H]UTP using a DNA template containing a T7 promoter and the luciferase gene (pT7Luc, Fig. 3) (Fig. 2A). A high level of RNA polymerase activity was demonstrated in all of the cell lines examined. Polymerase activity increased with the increase in m.o.i. The cells infected with AdexCAT7 at an m.o.i. of 100 showed CPE, therefore m.o.i. 20 was employed throughout the experiments. Mammalian cells infected with AdexCAT7 were harvested periodically and examined for their T7 polymerase expression. Western blot analysis revealed continuous T7 polymerase expression for at least 11 days postinfection in HepG2 and HeLa cells (Fig. 2B). Cells did not show any evidence of cytotoxicity during this period. Similar results were also obtained in other cell lines (data not shown). No polymerase activity was detected in cells infected with a control virus containing only the promoter sequence (AxCAwt).

Transcription of RNA from reporter plasmids

We constructed three reporter plasmids for *in vivo* RNA synthesis (Fig. 3). The pT7HCVLuc plasmid possesses a modified T7 promoter and transcription was expected to start at the last G residue of the 3′ end of the promoter. Therefore, RNA synthesized from the plasmid should have an identical sequence with that of the 5′UTR of HCV. pT7Luc has the luciferase gene directly under the control of the authentic T7 promoter. pT7EMCVLuc carries the 5′UTR sequence of encephalomyocarditis virus (EMCV) preceding the luciferase gene, which is expressed under the control of the authentic T7 promoter. In pT7EMCVLuc and pT7Luc, transcription should start efficiently from the third nucleotide upstream of the 3′ end of the authentic T7 promoter (Fig. 3, bottom line). Therefore, both of the transcripts should have three extra G residues at the 5′ end. Transcriptional efficiency of pT7HCVLuc may thus be lower than that of pT7EMCVLuc and pT7Luc because of the deletion of the last two G residues from the promoter sequences (Milligan *et al.,* 1987).

The transcription efficiencies of these three reporter plasmids were then compared in various cells infected with AdexCAT7. Cells were harvested 6 h after transfection, and transcription levels were measured by Northern blot analysis (Fig. 4). Efficient transcription was observed in cells transfected with pT7EMCVLuc and pT7Luc. However, in HepG2 and Huh7 cells transfected with pT7HCVLuc, no transcripts were detected. Similar results were obtained in COS7, HeLa, and CPK cells (data not shown). Only in FLC4 cells were transcripts derived from pT7HCVLuc detected, although the amounts of the transcript were smaller than those derived from pT7EMCVLuc and pT7Luc. These results suggest that the efficiency of transcription from pT7HCVLuc was enhanced in FLC4 cells.

Determination of terminal sequences of HCV minigene RNA

The above results indicated that HCV minigene RNA was transcribed well in FLC4 cells. Further experi-

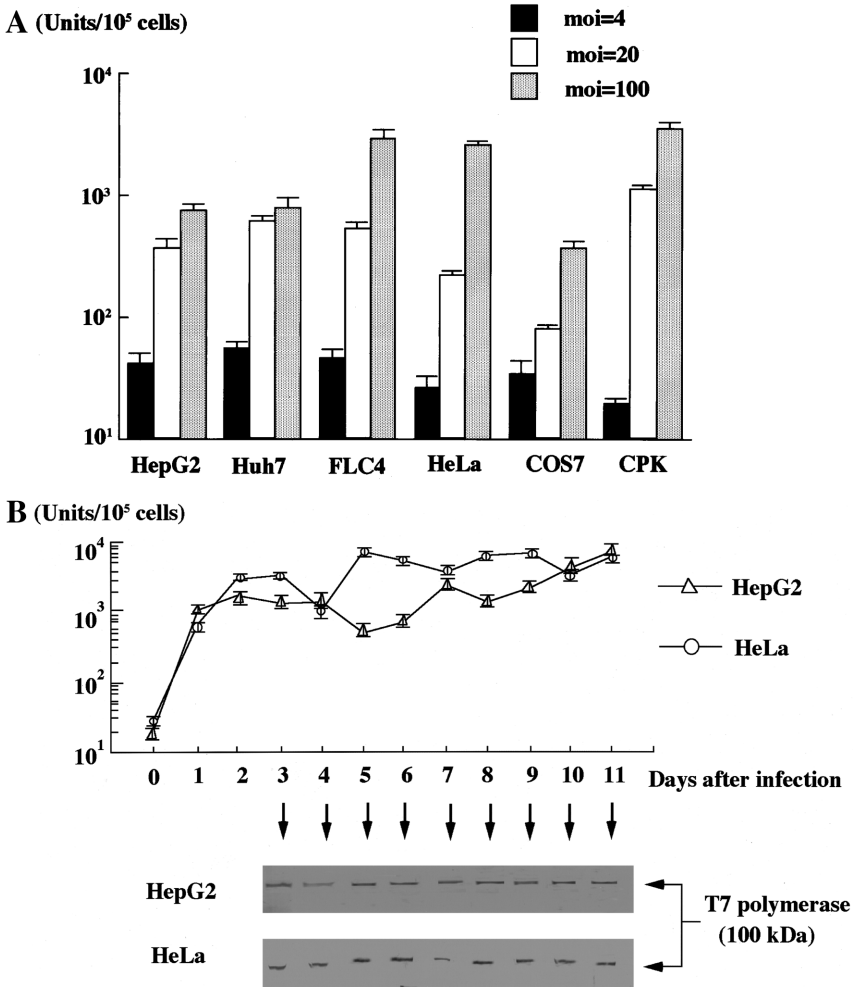

FIG. 2. Expression of T7 polymerase in cells infected with AdexCAT7. (A) T7 RNA polymerase activity in cell lysates was measured with a DNA template containing a T7 promoter. Cells were infected with AdexCAT7 at m.o.i.s of 4, 20, and 100. T7 RNA polymerase activity was assayed as described under Materials and Methods. Results represent the means of three independent experiments. Standard deviations are represented by vertical lines. (B) Time course of T7 RNA polymerase expression in HepG2 and HeLa cells infected with AdexCAT7. Cells were infected with AdexCAT7 at an m.o.i. of 20 and harvested at different times. Results represent the means of three independent experiments. Standard deviaitons are represented by vertical lines. Expression of T7 polymerase was also determined by immunoblotting. The arrows indicate the T7 polymerase proteins.

ments were then performed in FLC4 cells. Previous reports of the successful construction of a full-length cDNA clone of a flavivirus indicated that authenticity of the nucleotide sequences at the 5′ end of the transcripts was crucial for recovery of the infectious virus (Rice *et al.,* 1989; Lai *et al.,* 1991; Sumiyoshi *et al.,* 1992; Boyer and Haenni, 1994; Khromykh and Westaway, 1994). Consequently, it was important to determine the precise sequence of the 5′ terminal region of the HCV minigene RNA. A 5′ RACE method was used to amplify the 5′ end of the transcript, and then the 5′UTR sequence (position 1–207 nt) was analyzed by PCR-based direct sequencing. The 3′ terminus structure was determined by the oligo RNA ligation method. RNA derived from pT7HCVLuc started from the expected G residue and terminated after poly(T_{15}). This structure is identical to the HCV minigene sequence in pT7HCVLuc used for transfection (data not shown).

Expression of luciferase after transfection of AdexCAT7-infected cells with the reporter plasmids

Cells infected with AdexCAT7 at an m.o.i. of 20 were transfected with the reporter plasmids and examined for luciferase activity 24 h after transfection (Fig. 5). The highest level of luciferase activity was detected in all the cells transfected with pT7EMCVLuc, whereas the lowest luciferase activity was recovered in cells transfected with pT7Luc. The luciferase activity in cells transfected with pT7HCVLuc except for FLC4 cells was 70- to 140-fold lower than pT7EMCV. However, luciferase activity in FLC4 cells transfected with pT7HCVLuc was only three times lower than that of pT7EMCVLuc-transfected cells despite the lower efficiency of transcription due to the modification of the T7 promoter (Fig. 4). These results further suggested that translation efficiency derived from pT7HCVLuc or

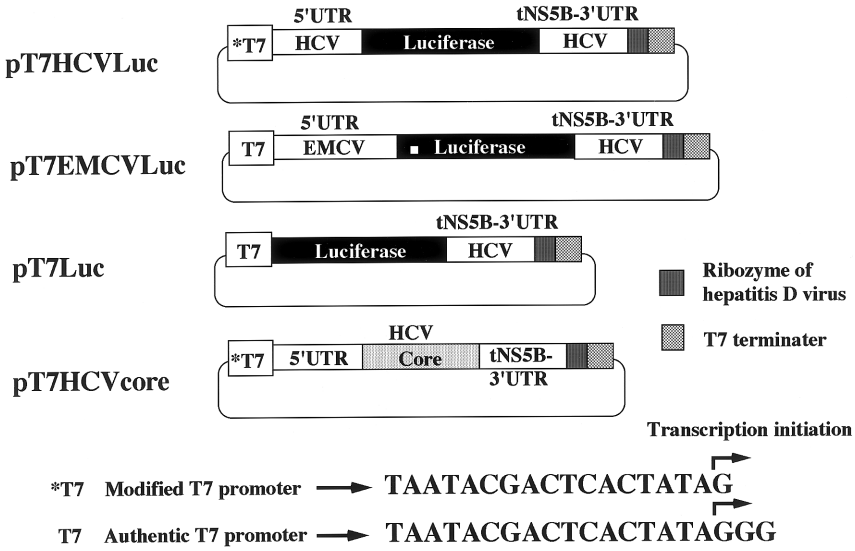

FIG. 3. Structure of reporter plasmids. Reporter plasmids were constructed as described under Materials and Methods. Nucleotide sequences of authentic and modified T7 promoters and transcription intiation sites of the promoters are indicated at the bottom.

stability of the transcript derived from pT7HCVLuc was significantly increased in FLC4 cells.

Expression of luciferase after transfection with RNA synthesized *in vitro*

To examine the possibility that the efficiency of translation of RNA possessing the 5′UTR of HCV is higher than the EMCV-dependent translation in FLC4 cells, different cell lines were transfected with RNAs synthesized from the respective reporter plasmids *in vitro* and examined for luciferase activity 4–48 h after transfection (Fig. 6). RNA derived from pT7EMCVLuc showed a similar pattern in all three cell lines examined; the highest luciferase activity was observed 4–12 h after transfection and then gradually decreased. In HeLa and HepG2 cells transfected with RNA derived from pT7HCVLuc, results similar to those from pT7EMCVLuc were obtained. In contrast, two- to three-fold higher luciferase activity was detected in FLC4 cells transfected with RNAs derived from pT7HCVLuc than from pT7EMCVLuc. In FLC4 cells transfected with HCV minigene RNA, luciferase activity was highest 12 h after transfection and continued until at least 48 h after transfection (Fig. 6). These results suggested the presence of host cell factor(s) in FLC4 cells that play some role in increasing the stability of the HCV minigene RNA and/or the efficiency of translation of the RNA.

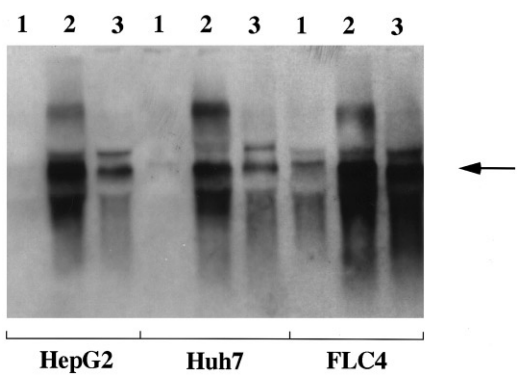

FIG. 4. Transcription of reporter plasmids *in vivo*. Northern blot analysis of RNA synthesized in cells infected with AdexCAT7 and transfected with pT7HCVLuc (lane 1), pT7EMCVLuc (lane 2), or pT7Luc (lane 3) 24 h after infection. At 6 h after transfection, RNA was extracted and examined for transcription of the plasmids by Northern blot analysis as described under Materials and Methods. The arrow indicates the RNAs synthesized in cells.

Expression of HCV core protein in FLC4 cells by the T7-adenovirus system

To determine whether HCV proteins can be synthesized in cells by the T7-adenovirus system, we constructed a plasmid, pT7HCVcore, containing the modified T7 promoter, the HCV 5′UTR, the HCV core gene, the coding region of C-terminal of NS5B and the HCV 3′UTR, rHDV and the T7ter (Fig. 3). FLC4 cells infected with AdexCAT7 were transfected with pT7HCVcore and examined for expression of core protein by Western blotting. The HCV core protein was detected in cell lysates prepared 1–3 days after transfection. No core protein was expressed in cells infected with the control adenovirus, AxCAwt (Fig. 7).

DISCUSSION

In this study, we established a system to express the bacteriophage T7 RNA polymerase by using a replication-defective recombinant adenovirus. By transfection of various mammalian cells infected with AdexCAT7 with a plasmid harboring a minigene of HCV under the modified T7 promoter, one human hepatoma cell line, FLC4, was

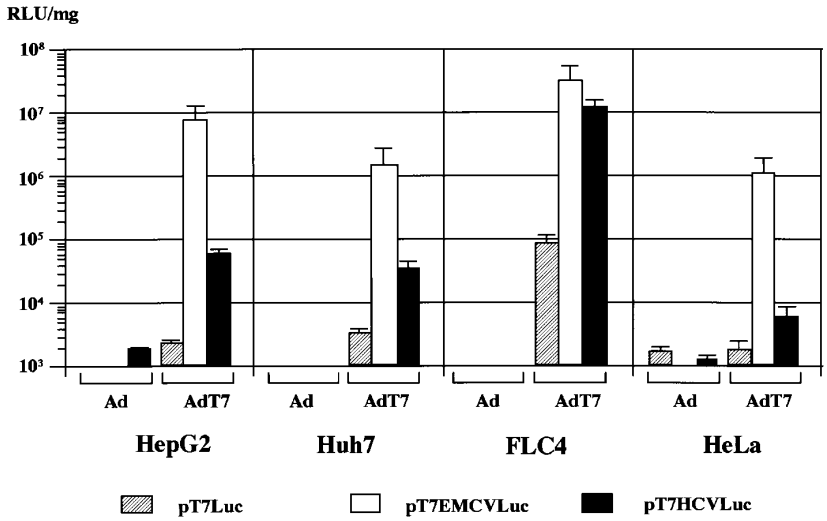

FIG. 5. Expression of reporter plasmids in cells infected with AdexCAT7. Cells were infected with AdexCAT7 or AxCAwt at an m.o.i. of 20 and transfected with reporter plasmids 24 h after infection. The luciferase activity was measured 24 h after transfection. The firefly luciferase activity is expressed as relative light units (RLU) per mg of protein after normalization with that of the *Renilla* luciferase used as an internal standard. Results represent the means of three independent experiments. Standard deviations are represented by vertical lines. Ad: AxCAwt, AdT7: AdexCAT7.

found to increase the stability and the translational efficiency of the RNA.

A vaccinia virus vector expressing T7 RNA polymerase has been used for transient viral gene expression (Fuerst *et al.,* 1987). This VacT7 system was particularly effective when the virus under study did not have an efficient cell-culture system for replication. The VacT7 system has contributed significantly to the understanding of the processing of HCV polyproteins (Kohara *et al.,* 1992; Spaete *et al.,* 1992; Grakoui *et al.,* 1993; Selby *et al.,* 1993; Tomei *et al.,* 1993; Manabe *et al.,* 1994). Furthermore, the VacT7 system has been used in the recovery of infectious RNA viruses from their cDNA clones (Conzelmann and Schnell, 1994; Collins *et al.,* 1995; Garcin *et al.,* 1995; Lawson *et al.,* 1995; Whelan *et al.,* 1995; Kato *et al.,* 1996). The major benefit of this system is its broad host range, which permits foreign gene expression in many mammalian cells. However, this system is not suitable for long-term analysis of the expressed products because of its extensive CPE, and therefore it cannot be used to recover viruses like HCV whose growth is slow and inefficient. Extensive CPE caused by the vaccinia virus itself hampers the *bona fide* virus–cell interaction. In this context, it is noteworthy that an avian host restricted vaccinia

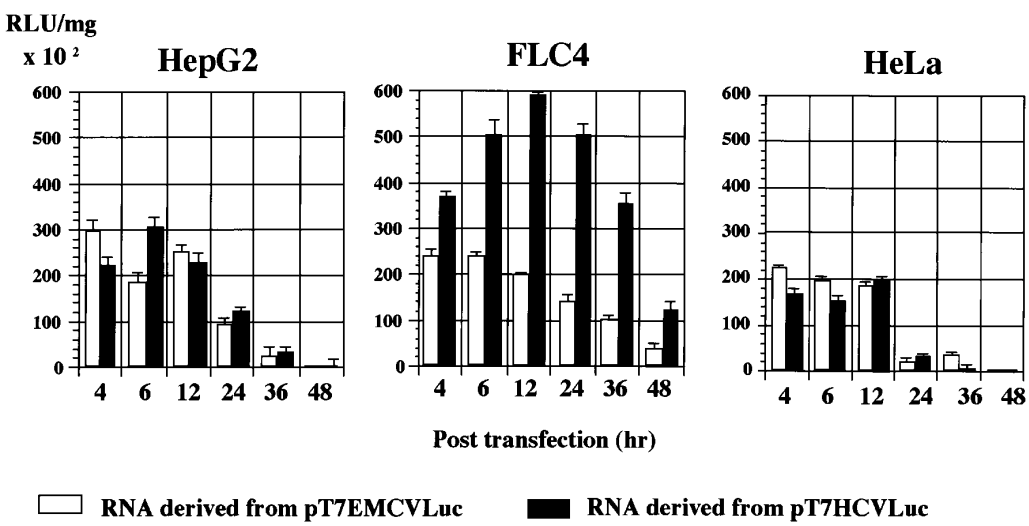

FIG. 6. Expression of luciferase in cells transfected with RNA. Cells were transfected with RNA transcripts of pT7EMCVLuc or pT7HCVLuc. The cells were examined for luciferase activity at various time points after transfection. Luciferase activity is expressed as RLU per mg of protein after normalization with that of the *Renilla* luciferase used as an internal standard. Results represent the means of three independent experiments. Standard deviations are represented by vertical lines.

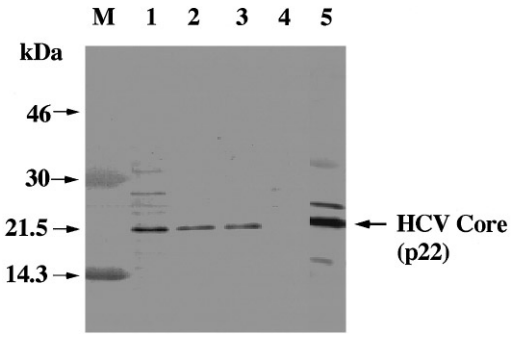

FIG. 7. Expression of HCV core protein in FLC4 by the T7-adenovirus system. FLC4 cells were infected with AdexCAT7 (lanes 1–3) or AxCAwt (lane 4) at an m.o.i. of 20. At 24 h after infection, cells were transfected with pT7HCVcore and harvested 24 h (lanes 1 and 4), 48 h (lane 2), and 72 h (lane 3) after transfection. As a positive control HCV core proteins expressed in insect cells by a recombinant baculovirus, Ac39 (Suzuki *et al.,* 1995), was used (lane 5). The expressed core protein was detected by Western blot analysis. M is a molecular weight marker.

virus, strain Ankara, has been developed which is less cytopathic for many cell types (Wyatt *et al.,* 1995).

Recently, Tomanin *et al.* reported the construction of replication competent and deficient adenoviruses possessing the T7 RNA polymerase gene under the control of the human cytomegalovirus promoter (Tomanin *et al.* 1997). Although the replication-competent virus exhibited a high level of T7 polymerase expression in various cell lines, low-level expression was detected in cell lines other than 293 after infection with the replication-deficient recombinants. After coinfection with replication-deficient adenoviruses possessing the T7 polymerase gene and the reporter gene under the control of the T7 promoter linked to the internal ribosome entry site (IRES) of EMCV, only low or no reporter activity was detected in replication-incompetent cell lines. In contrast, the T7-adenovirus constructed in our study exhibited not only a high level but long-lasting expression of T7 RNA polymerase in various mammalian cells without CPE. This might be due to the efficiency of the promoter used in the recombinant viruses. By using a ubiquitously strong promoter, it may be possible to establish a versatile expression system for various cell lines. We demonstrated here that this system is applicable to a wide variety of mammalian cells. Foreign genes were efficiently expressed under the control of the T7 promoter in these cells. The T7 RNA polymerase *per se* has no capping activity and is localized in the cytoplasm. Therefore, RNA synthesized by this system should be uncapped and present in the cytoplasm. Consequently, an IRES is required for gene expression in this system. This is in contrast to the VacT7 system in which a portion of the RNA transcript was modified by the virus-coded capping enzyme (Elroy-Stein *et al.,* 1989). Conversely, the T7-adenovirus system may actually provide an advantage for generation of infectious RNA *in vivo* because of its lack of capping activity. Infectious cDNA clones have been successfully made for flaviviruses (Rice *et al.,* 1989; Lai *et al.,* 1991; Gristun and Gould, 1995; Sumiyoshi *et al.,* 1992). Authenticity of the sequence at the 5′ end of the transcripts was shown to be crucial for construction of infectious cDNA clones of these RNA viruses (Boyer and Haenni, 1994). Inappropriate capping at the 5′ end by vectors may thus hinder the authentic structure of the RNA and prevent its infectivity.

Many full-length cDNA clones of HCV have been obtained since the first report of cloning and characterization of the prototype HCV cDNA (Choo *et al.,* 1991; Han *et al.,* 1991; Inchauspe *et al.,* 1991; Kato *et al.,* 1990; Okamoto *et al.,* 1991; Tanaka *et al.,* 1996). In most cases, the starting materials were plasma pools of blood containing infectious HCV. The cDNA fragments were obtained by RT–PCR by using many sets of primers derived from the prototype HCV sequence. Therefore, it is not certain whether the obtained sequences originated from a single genome of an infectious virion of HCV. There is no sufficient system yet to evaluate the replication of HCV *in vitro* or to isolate an infectious particle from the quasi-species population of HCV. Therefore, it is impossible to assess the authenticity of the cDNA clones of HCV at present. Recently, it was demonstrated that a full-length HCV RNA transcribed from a consensus cDNA clone of HCV was infectious for a chimpanzee by direct injection into his liver. However, propagation of infectious RNA in cell culture has not been reported so far (Kolykhalov *et al.,* 1997; Yanagi *et al.,* 1997). In the case of an infectious cDNA clone of classical swine fever virus, only five amino-acid changes abrogated its infectivity (Moormann *et al.,* 1996).

Instead of full-length clones, establishment of a minigene of HCV may circumvent this difficulty. A modified T7 promoter in pT7HCVLuc was engineered to generate the authentic HCV 5′ end sequence of the transcript by deleting two Gs. Although deletion of two guanine residues in the transcription initiation site of the promoter decreases the efficiency of transcription in comparison with the authentic one (Milligan *et al.,* 1987), the expression of luciferase in FLC4 cells was significantly higher than in other cell lines examined. RNA possessing the 5′UTR sequence of EMCV is efficiently translated via direct entry of the ribosome at a landing site within the UTR (Bienkowska-Szewczyk and Ehrenfeld, 1988; Trono *et al.,* 1988; Jan *et al.,* 1989; Pelletier and Sonenberg, 1989)

Although only a faint signal of transcript from pT7HCVLuc was detected in FLC4 cells (Fig. 4), the level of luciferase activity in this cells transfected with pT7HCVLuc was comparable to in those transfected with pT7EMCVLuc (Fig. 5). Therefore, we would have expected the translational efficiency of the RNA possessing HCV 5′UTR in FLC4 cells to be enhanced or stabilized. The level of expression of luciferase observed in FLC4 cells transfected with the HCV minigene RNA was only two- to threefold higher than in those transfected with RNA carrying the IRES sequence of EMCV (Fig. 6). These results

suggest the presence of some host factor(s) which increase the efficiency of translation of the HCV minigene RNA in FLC4 cells. Identification of such host factor(s) and elucidation of the mechanism of the enhancement of translation of the HCV minigene RNA in this cell line are now in progress.

We designed the pT7HCVLuc construct so that the ribozyme sequence of hepatitis D virus may cleave synthesized RNA immediately after the terminal poly (U) sequence. However, the presence of an additional 98 nt of HCV sequence downstream of 3′ poly (U) tract was recently reported (Kolykhalov *et al.,* 1997; Tanaka *et al.,* 1996). We also detected the additional 98-nt sequence in both positive and negative strands of HCV RNA (Aizaki *et al.,* 1998). The effect of the 3′ terminal sequence on transcriptional or translational efficiency in this system must be determined. So far, however, no difference in reporter gene expression was observed by the addition of the 98-nt extra sequence in the 3′ end of the pT7HCVLuc in this system (data not shown). Studies of replication of the HCV minigene RNA by supplementing HCV nonstructural proteins with infection with adenovirus vectors are also in progress. The *in vivo* system used to synthesize the HCV minigene described in this study could offer an efficient tool for studies not only of HCV replication but also of other viruses for which there are no efficient *in vitro* replication systems.

MATERIALS AND METHODS

Cells

Cells were maintained in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, NY) containing 2 mM L-glutamine, penicillin (50 IU/ml), streptomycin (50 μg/ml) and supplemented with 10% fetal calf serum. Human hepatocellular carcinoma cell lines (HepG2 and Huh7) were obtained from the American Type Culture Collection and the Japanese Cancer Research Resources Bank (JCRB)-Cell, Tokyo, respectively. A porcine kidney cell line (CPK) (Komaniwa *et al.,* 1981) was kindly provided by Dr. A. Fukusho, National Institute of Animal Health, Tokyo, Japan. The FLC4 cell lines was established by cloning JHH-4 cells (Sujino *et al.,* 1986), and possesses normal liver functions. The 293 cell line (Graham *et al.,* 1977) was used for propagation and titration of recombinant adenoviruses.

Construction of a recombinant adenovirus

The bacteriophage T7 RNA polymerase gene was excised from pVR-T7–1 (kindly provided by Dr. M. Kohara) with *Sma*I and *Eco*RI, filled with the Klenow enzyme and cloned into the *Swa*I site of transfer cosmid pAxCAwt (Kanegae *et al.,* 1995). The resultant cosmid was designated pAdexCAT7. The pAxCAwt is a cassette cosmid containing the CAG promoter and an almost full length adenovirus type 5 genome with deletions in the E1 (nt 453–3328) and E3 (nt 28592–30474) regions. The CAG promoter (Niwa *et al.,* 1991), is a composite promoter consisting to the cytomegalovirus IE enhancer, the chicken β-actin promoter and the rabbit β-globin polyadenylation [poly(A)] signal. Recombinant adenovirus containing the bacteriophage T7 RNA polymerase, AdexCAT7, was constructed (Fig. 1) as described previously (Miyake *et al.,* 1996). Briefly, 293 cells were transfected with pAdexCAT7 together with the DNA-terminal protein complex of a parent virus by the calcium phosphate precipitation method (Sambrook *et al.,* 1989). Recombinant virus was plaque-isolated 10 days after transfection. Viral DNA was extracted together with cellular DNA, and the desired recombinant was identified by restriction analysis (Sambrook *et al.,* 1989). The AxCAwt virus (Kanegae *et al.,* 1995), which did not carry an expression unit, was used as a control virus. The recombinant viruses were extensively purified by banding twice through CsCl density gradients and dialyzed against buffer containing 10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, and 10% glycerol.

Construction of plasmids

The HCV cDNAs (genotype 1b) used in this study were originally isolated from a blood sample of an HCV carrier, which was infectious for both humans and chimpanzees (NIHJ1 clone) (Aizaki *et al.,* 1998).

To synthesize HCV minigene RNA, pT7HCVLuc (Fig. 3) was constructed as follows: first, to make pUCT707 containing a modified T7 promoter, the HCV 5′UTR and part of the core region, the clone pUCAB [containing 1–707 nt (Aizaki *et al.,* 1998)] was modified by the PCR by using 5′ primer, 5′-AAGCTTAATACGACTCACTAATAGCCAGCCCCCGATTGGGGGCGACACTCCAT-3′, which contains a *Hin*dIII site and the modified T7 promoter sequence fused directly to the 5′ end of the HCV sequence (underlined), and 3′ primer, 5′-TTGTGATGAGCCGATCGTCA-3′. To generate pUCT7, 5′UTRLuc containing the modified T7 promoter, the 5′UTR and the firefly luciferase gene, pUCT707 was digested with *Cla*I, filled with Klenow, and ligated with an 8-mer *Bam*HI linker (5′-CGGATCCG-3′). After *Bam*HI and *Hin*dII digestion, the fragment was cloned into the *Hin*dIII-*Bam*HI site of the PicaGene vector (Toyo Ink, Tokyo, Japan) just upstream of the luciferase gene. To remove the redundant sequences between the 5′UTR of HCV and the luciferase gene, the *Acc*I-*Kas*I fragment of the plasmid was replaced with a DNA fragment synthesized by two oligonucleotides, 5′-TAGACCGTGCATCATGGAAGACGCCAAAAACATAAAGAAAGGCCCGGCGCCATTC-3′ (top strand) and 5′-GAATGGCGCCGGGCCTTTCTTTATGTTTTTGGCGTCTTCCATGATGCACGGTCTA-3′ (bottom strand), which contains an *Acc*I site (327 nt of HCV) at the 5′ end and a *Kas*I site (30 nt of the luciferase gene) at the 3′ end. Thus the initiator AUG of

the luciferase gene in pUCT7 5′UTRLuc was located 342 nt downstream from the transcription initiation site which is identical to the coding sequence of the HCV core protein. To modify the 3′ end of pUCT7 5′UTRLuc, the *Bsp*MI-*Hin*dIII fragment (nt 9354–9425) of pUCJK (Aizaki *et al.,* 1998) was replaced with a PCR fragment obtained by using a set of primers, 5′-GCGTCCCAGCTGGACTT-GTC-3′ and 5′-TTCGAAATTTTTTTTTTTTTTTCCCCTACC-GGATAACCGGACC-3′, which contained 15 nt of poly (U) stretch upstream of the *Dra*I site. The cDNA of the modified pUCJK was digested with *Asp*718, filled with Klenow enzyme and ligated with an 8-mer *Xho*I linker (5′-CCTC-GAGG-3′). The two fragments, the *Hin*dIII-*Xho*I fragment of pUCT7 5′UTRLuc and the *Xho*I-*Dra*I fragment of modified pUCJK, were cloned between the *Hin*dIII-*Sma*I sites of pX8dT (kindly provided by Dr. K. Conzelmann), which is a derivative of pBluescriptII SK(−) (Stratagene, La Jolla, CA), and the resultant plasmid was designated as pT7HCVLuc. pT7HCVLuc therefore contained the modified T7 promoter, the HCV 5′UTR (341 nt), the luciferase gene, a part of NS5B, the HCV 3′UTR, a poly (T_{15}) sequence, a hepatitis D virus ribozyme sequence (rHDV), and a T7 promoter termination sequence (T7ter). Transcription of pT7HCVLuc by T7 polymerase was considered to be initiated from the G residue identical to the first nucleotide of the 5′ end of the HCV and would be terminated after poly (U_{15}) tailing by self-cleavage activity of rHDV.

As a positive control, pT7EMCVLuc was constructed (Fig. 3). This plasmid was constructed by replacing the T7 promoter and 5′UTR of the pT7HCVLuc with the authentic T7 promoter and the 5′UTR of EMCV derived from pTM3 (kindly provided by Dr. B. Moss). As a negative control, pT7Luc was constructed by deleting the HCV 5′UTR derived from pT7HCVLuc and by replacing the modified T7 promoter with the authentic one (Fig. 3).

To express HCV core protein using the T7-adenovirus system, pT7HCVcore was constructed (Fig. 3). This plasmid contains the modified T7 promoter, the HCV 5′UTR, the HCV core region, a part of NS5B, the HCV 3′UTR, the poly (T_{15}) sequence, rHDV, and T7ter. To normalize the transfection efficiency, pT7EMCVRLuc was constructed and used as an internal control plasmid. *Renilla* luciferase gene was excised from pRL-null (Promega, Madison, WI) and inserted into the *Nco*I site of pTM3 under the authentic T7 promoter and the 5′UTR of EMCV.

T7 RNA polymerase assay

Two $\times$ 10^5 cells in a 12-well plate were infected with AdexCAT7 at m.o.i.'s of 4, 20, and 100. After 48 h, the cells were washed three times with phosphate-buffered saline (PBS), resuspended in 200 μl of PBS and sonicated for 5 min at 30-s intervals. After centrifugation, the cell lysates were used for *in vitro* polymerase as follows: a total of 20 μl of a reaction solution containing 1 μg of DNA template (pT7Luc, Fig 3), 2 μl of lysate, 2 μl of transcription buffer (MEGAscript kit, Ambion, Austin, TX), and 0.15 mM each of ATP, CTP, GTP, UTP, and labeled [^{3}H]UTP (37 KBq) (Daiichi Kagaku, Tokyo) was incubated at 37°C. Fifty units of enzyme mix containing purified T7 RNA polymerase (Promega) and 2 μl of lysates of uninfected cells was used as positive and negative controls, respectively. After 3 h of incubation, 8 μl of the reaction solution was spotted onto a filtermat (Wallac, Turku, Finland) and dried completely in an 80°C oven, then washed three times with 2$\times$ standard saline citrate (0.3 M sodium chloride and 0.03 M sodium citrate) and twice with 95% ethanol. After drying, a Meltilex (Wallac) sheet was laid directly on the dried filtermat and melted at 80°C for 5 min. Subsequently the filtermat with meltilex was removed from the oven, solidified at room temperature and finally placed in a microbeta counter (1450 Microbeta Plus, Wallac) for determination of radioactivity.

Expression of luciferase in cells infected with AdexCAT7

Two $\times$ 10^5 cells of various cell lines in a 12-well plate were infected with AdexCAT7 at an m.o.i. of 20. The inocula were removed after 60 min of incubation and replaced with 1 ml of fresh medium. After 24 h of incubation, the medium was removed, the cells were washed twice and the medium was replaced with 500 μl of Opti-MEM-medium (GIBCO BRL, Life Technologies, Gaithersburg, MD). Reporter plasmid containing firefly luciferase gene (1 μg), pT7EMCVRLuc containing *Renilla* luciferase gene as an internal control plasmid (0.1 μg) and lipofectin (GIBCO BRL) (2.5 μl) were mixed with 50 μl of Opti-MEM and the infected cells were transfected with the mixture after 15 min of incubation. The cells were examined for expression of reporter genes at 24 h after transfection. Firefly and *Renilla* luciferase activities were determined by the dual-luciferase reporter assay system (Promega) following the manufacture's instructions. Briefly, cells were washed twice with PBS and lysed with passive lysis buffer (Promega). After centrifugation, the cell lysates were used for the assay of two luciferase reporter enzymes. Since the firefly and *Renilla* luciferases have dissimilar enzyme structures and substrate requirements, it is possible to selectively discriminate between their respective bioluminescent reactions. The firefly luciferase reporter assay was initiated by adding the cell lysates to luciferase assay buffer (Promega). Then to quench of firefly luciferase luminescence and simultaneously activate the luminescent reaction of *Renilla* luciferase, Stop and Glo Buffer (Promega) was added to the same tube. Relative light units (RLU) were measured with a luminometer (Berthold, Wildbad, Germany), and firefly luciferase activity was normalized with that of *Renilla* luciferase. Total protein was quantified by

using BCA protein assay reagents (Pierce Chemical Co., Rockford, IL).

Northern blot analysis

Total RNA was prepared from cells infected with AdexCAT7 and then transfected with the reporter plasmids by RNA zol B (Cinna/Biotecx, Friendswood, TX). RNA was denatured at 65°C for 15 min in 5.8 μl of sample buffer (17.5% formaldehyde and 50% formamide in 1× MOPS buffer, 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA pH 7.0). After cooling in ice-cold water, the RNA in 7.5 μl of 2× loading buffer (80% formamide, 0.1% bromphenol blue, 0.1% xylene cyanol, 2 mM EDTA) was separated on a formaldehyde-denatured agarose gel (18% formaldehyde, 1% agarose) in 1× MOPS buffer. The gel was washed twice with 20× SSC (3 M NaCl, 0.3 M trisodium citrate dihydrate, pH 7.0) for 15 min and transferred onto a Hybond N^+ membrane filter (Amersham Life Science, Buckinghamshire, England) by the capillary method. The filter was dried and RNAs were fixed on the membrane with UV light (UV Crosslinker, Funakoshi, Tokyo). The RNA was randomly labeled with digoxigenin (DIG)-UTP *in vitro* using the luciferase gene as a template. Luciferase-specific RNA was detected with a DIG luminescent detection kit (Boehringer GmbH, Mannheim, Germany) according to the manufacturer's protocol.

The nucleotide sequences of the 5′ and 3′ UTRs of the HCV minigene RNA using AdexCAT7

Total RNA was extracted from cells infected with AdexCAT7 and then transfected with pT7HCVLuc as described above. The nucleotide sequence of the 5′ terminus position was determined after amplification of the 5′ terminus region using the 5′ RACE for rapid amplification of cDNA ends kit (GIBCO BRL). Briefly, first strand cDNA was synthesized from total RNA using a 20-mer primer, Aa1 (5′)-GTTGATCCAAGAAAGGACCC-3′) (Aizaki *et al.,* 1998) using reverse transcriptase (Takara Shuzo, Kyoto). A homopolymeric tail, using dCTP and terminal deoxyl terminal transferase, was then added to the 3′ end of cDNA. The 5′ terminus sequence (position 1 to 207) was determined by PCR-based sequencing using the M13RV (5′-CAGGAAACAGCTATGAC-3′) and J36A primers.

The nucleotide sequence of the 3′ terminus position was determined by the oligo RNA ligation method (Tanaka *et al.,* 1996). Briefly, the 5′-phosphorylated oligo RNA (5′-CCCGUCUUCGGACAUCCAGAGGUAU-3′) was synthesized (Takara Shuzo) and ligated to RNA by T4 RNA ligase (Takara Shuzo) at 10°C for 15 h and then reverse transcribed with primer La1 (5′-CCATCCTAATACGACTCACTATAGGGC-3′) at 50°C for 1 h. The reverse transcript was amplified by nested PCR (94°C for 1 min, 55°C for 1 min, 72°C for 2 min, 40 cycles) with sets of the primers (1st PCR: Ks1; 5′-TACTCCTACTCTCTGTAGGG-3′ and La1; 2nd PCR: Ks2; 5′-GGCATCTACCTGCTCCCCAA-3′ and La2; 5′-ACTCACTATAGGGCTCGAGCGGC-3′). The 3′ terminus sequences were determined by PCR-based sequencing

In vitro transcription

RNAs were synthesized with a MEGAscript kit according to the manufacturer's instruction. Briefly, the transcription reaction was carried out at 37°C for 2 h in a total 20 μl reaction solution containing 1 μg of DNA template, 2 μl of 10× transcription buffer, 2 μl of enzyme mix, 2 μl of 10× transcription buffer, 2 μl each of 75 mM ATP, CTP, GTP, and UTP. The mixture was treated twice with 8 units of DNase at 37°C for 20 min and precipitated with ethanol. After being washed with 75% ethanol, the precipitate was dissolved in water.

RNA transfection

Cells (2×10^5) in a 12-well plate were washed twice with 500 μl of Opti-MEM (GIBCO BRL) medium and replaced with the same volume of Opti-MEM medium. *In vitro*-synthesized RNA (2 μg) and lipofectin (GIBCO BRL) (2.5 μl) were mixed with 50 μl of Opti-MEM, and cells were transfected with the mixture after 15 min of incubation. To normalize the transfection efficiency, RNA synthesized from pT7EMCVRLuc was used as an internal control. At 6 h after transfection, the cells were harvested for luciferase assay as described above.

Western blot

Cell extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and proteins were blotted onto a polyvinylidene difluoride membrane (Millipore, Tokyo). Anti-T7 polymerase rabbit polyclonal antibody (kindly provided by Dr. F. W. Studdier) and a mouse monoclonal antibody raised against the core protein of HCV (Suzuki *et al.,* 1995) were used as the first antibody. The expressed proteins were detected with an alkaline phosphatase assay kit (Bio-Rad Laboratories, Hercules, CA).

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